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Chromosomal deletions are very common events in breast cancer. However, no TSGs have been identified from most of recurrent deletions and few identified TSGs do not account for the risk of majority of breast cancer. In additional to the classical TSGs, there are haplo-insufficient TSGs which defy the identification through mutation analysis and may be quite common. By using a new system to generate random chromosomal deletions, we identified a ~3Mbp deletion in mouse chromosome 3, which was associated with tumorigenesis. The expression of Fat4 in the deleted region was inactivated due to promoter methylation in the second allele of Fat4, and the re-expression of Fat4 suppressed the tumorigenecity, suggesting Fat4 as a strong candidate for breast tumor suppressor genes. We also found that Fat4 expression was lost in a high proportion of human breast cancers, some of which were attributed to Fat4 promoter methylation.

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INTRODUCTION

Chromosomal deletions are very common events in breast cancer. Analysis of recurrent deleted regions helps to identify tumor suppressor genes, based on Knudson's two-hit model which defines a tumor suppressor gene (TSG) by finding mutations or deletions in both alleles of a gene in tumors. However, no TSGs have been identified from most of recurrent deletions and few identified TSGs do not account for the risk of majority of breast cancer. In additional to the classical TSGs, there are haplo-insufficient TSGs which defy the identification through mutation analysis and may be quite common. Moreover, as chromosomal deletions in breast tumors are often large and encompass many genes, one deletion may include two or more related genes, a combined haplo-insufficient effect of which could contribute to the development of breast cancer. By using a system to generate random chromosomal deletions, we proposed to identify these haplo-insufficient TSGs. The identification of new TSGs is crucial for our understanding of breast cancer biology and breast cancer management

BODY

Task 1. Identify the chromosome regions causing transformation of the non-transformed, immortal breast cells when deleted.
By using a random chromosome deletion method based on LoxP/Cre mediated homologous recombination, we identified a ~3Mbp deletion in mouse chromosome 3, which was associated with tumorigenesis of a non-tumorigenic mammary epithelial cell line.

Task 2. Determine which gene/genes are TSGs in the identified chromosome region.

We found that the expression of Fat4, one member of the Fat family, in the deleted region was inactivated due to promoter methylation in the second allele of Fat4, and the re-expression of Fat4 suppressed the tumorigenecity, suggesting Fat4 a strong candidate for a breast tumor suppressor gene. We also found that Fat4 expression was lost in ~60% of human breast tumor cell lines (6/10) and primary tumors (14/23). Loss of Fat4 expression in some of breast tumor cell lines was associated with Fat4 promoter methylation.

KEY RESEARCH ACCOMPLISHMENTS

* We identified a ~3Mbp deletion in mouse chromosome 3, which was associated with tumorigenesis.

* The expression of Fat4 in the deleted region was inactivated due to promoter methylation in the second allele of Fat4, and the re-expression of Fat4 suppressed the tumorigenecity, suggesting Fat4 as a strong candidate for breast tumor suppressor genes.

* We found that Fat4 expression was lost in a high proportion of human breast cancers, some of which were attributed to Fat4 promoter methylation

REPORTABLE OUTCOMES

(1). Qi C, Zhu YT, Hu L, Zhang Z, Rao SM, and Zhu YJ. Identification of Fat4 as the candidate tumor suppressor gene in breast cancers (submitted).

CONCLUSIONS

We identified a ~3Mbp deletion in mouse chromosome 3, which was associated with tumorigenesis. The expression of Fat4 in the deleted region was inactivated due to promoter methylation in the second allele of Fat4, and the re-expression of Fat4 suppressed the tumorigenecity, suggesting Fat4 as a strong candidate for breast tumor suppressor genes. We found that Fat4 expression was lost in a high proportion of human breast cancers, some of which were attributed to Fat4 promoter methylation

REFERENCES

(1). Qi C, Zhu YT, Hu L, Zhang Z, Rao SM, and Zhu YJ. Identification of Fat4 as the candidate tumor suppressor gene in breast cancers (submitted).

APPENDICES

One manuscript is attached.

Identification of Fat4 as a candidate tumor suppressor gene in breast cancers through random chromosome deletion

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ABSTRACT

A random chromosome deletion method based LoxP/Cre mediated homologous recombination was developed and used to isolate tumor suppressor genes for breast cancer. We identified a ~3Mbp deletion in mouse chromosome 3, which was associated with tumorigenesis of a non-tumorigenic mammary epithelial cell line. The expression of Fat4, one member of the Fat family, in the deletion region was inactivated due to promoter methylation in the second allele of Fat4, and the re-expression of Fat4 suppressed the tumorigenecity, suggesting Fat4 a strong candidate for a breast tumor suppressor gene. We also found that Fat4 expression was lost in ~60% of human breast tumor cell lines (6/10) and primary tumors (14/23). Loss of Fat4 expression in some of breast tumor cell lines was associated with Fat4 promoter methylation. In addition, we determined that loss of Fat4 expression led to a decreased expression of TP53 apoptosis effector (Perp), and an increased expression of matrix metalloproteinase 2 (MMP-2) and Hepatocyte growth factor (HGF), which have important roles in controlling epithelial integrity, cell migration, and cell proliferation.

INTRODUCTION

Loss-of-function mutations of the Fat gene in *Drosophila* causes hyperplasia of the pupal imaginal disks (26), suggesting that Fat is a candidate tumor suppressor gene. Excessive cell proliferation occurs with the loss of Fat expression while maintaining normal epithelial organization and differentiation potential (15). Overgrowth and abnormal cell behavior of Fat deficient cells during proliferation seem to be related to failures in cell signaling and/or cell adhesion (4). Fat belongs to the cadherin family that is involved in cell adhesion and consists of more than 80 members in mammalian species (40, 42, 44). The classic cadherins are Ca^{2+}-dependent cell-cell adhesion proteins characterized by five repeated cadherin-specific motifs in their extracellular domain (39). This motif is an approximately 110-amino-acid peptide that mediates homophilic interactions with other cadherin molecules, forming dimers which then interact with dimers on neighboring cells (39). Fat contains 34 cadherin motifs, four EGF-like repeats, a transmembrane domain, and a cytoplasmic region. Fat is involved in the planar polarity formation, which refers to the asymmetry of a cell within the plane of the epithelium (45). The second Fat gene in *Drosophila*, termed Fat-like, is involved in the formation of tubular organs (6).

The signaling pathway involved in the physiological and pathological function of Fat is largely unknown. The cytoplasmic domain of *Drosophila* Fat was found to interact with atrophin (12), which is a transcriptional co-repressor (48) and is the D*rosophila* homolog of human atrophins consisting of two members, Atrophin-1 (24, 30) and Atrophin-2 (50) (also known as Arginine (R) Glutamic Acid (E) Repeat Encoding or RERE). Atrophin-1 causes dentatorubral-pallidoluysian atrophy (DRPLA), a human neurodegenerative disease when its polyglutatmine tract is abnormally expanded (24, 30). Interestingly, Fat1 was recently shown to be processed with its cytoplasmic domain being released and translocated into nucleus (25).

There are four members of the Fat family in mice and humans (Fat1, MEGF1/Fat2, Fat3, and Fat4), which structurally resemble *Drosophila* Fat (11, 19, 29, 31). The tumor suppressor function is not observed with Fat1 as the null mutation of Fat1 in mice did not exhibit overgrowth of any organs (8). The knock out experiment did reveal novel roles of Fat1 in adhesion and cell-cell signaling. The function of the other three Fat genes remains to be investigated.

The identification of tumor suppressor genes has greatly advanced our understanding of breast cancer biology (32). Despite the progress, the total number of known tumor suppressor genes account for only a small fraction of familial breast cancer cases (32). In addition, the target genes for the vast majority of the loss of heterozygosity in breast cancers have not been revealed (10, 33). Therefore, many tumor suppressor genes must exist and remain to be discovered in breast cancers.

Cre is a recombinase which catalyzes the recombination between two LoxP sites - a 34-bp DNA element consisting of two 13 bp inverted repeats separated by an 8-bp spacer (36). If two LoxP sites are integrated into the DNA in the same direction, recombination catalyzed by Cre leads to the deletion of the interval DNA between the two LoxP sites. As the Cre is able to mediate the recombination of up to 5 megabase, the Cre-LoxP system has been adopted to create chromosome deletions (36, 49). Here, we report the development of a method to isolate the breast tumor suppressor gene. The method made use of the LoxP/Cre mediated homologous recombination to obtain random chromosome deletions in the genome. By applying this method to a mammary epithelial cell line, we identified Fat4, one member of the Fat family, as a strong candidate for a breast tumor suppressor gene. Furthermore, we found that Fat4 expression was lost in a large fraction of human breast tumors.

MATERIALS AND METHODS

Plasmids. To generate a synthetic intron carrying the 34 bp-LoxP site, PCR was performed with primers IntronA 5'-GCAGCAACAGATGGAAGGTGAGTACTCCCTCTCAAA-3' plus IntronB5'-CTGCCACACCTCAAGCTT-3' and primers IntronC 5'-AGCAGCTAGACTAGTGAGGTGTGGCAGGCTTGA-3' plus IntronD 5'-GTGCGGCGCCAGGAGGCCTGTGGAGAGAAAGGCAAA-3' using pIRES1neo (Clontech) as the template. The two PCR products were digested with HindIII and SpeI, respectively, which were then ligated to HindII-SpeI fragment containing a LoxP site from PBS246 (Invitrogen), followed by second round PCR using primers IntronA and IntronD. The synthesized intron was then inserted into the puromycin resistence gene (PAC) (between the first and second nucleotide of the codon encoding Gly^{111}) through PCR-based mutagenesis (Stratagene).

LacZ/Neo-5'Pur-LoxP-TK was generated by assembling EcoRI-XbaI fragment of a LacZ/Neo fusion cDNA, XbaI-BamHI fragment of 5'part of the puromycin resistance gene with a LoxP, and BglII-NotI fragment of the TK cDNA into the EcoRI/NotI sites of the vector PLIB vector (Clontech). Hyg-3'Pur-LoxP was created by inserting EcoRI-BglII fragment of the hygromycin resistance gene, and BamHI-ClaI fragment of 3'part of the puromycin resistance gene with a LoxP into the EcoRI/ClaI sites of the PLIB vector. All constructs were verified by sequencing.

Chromosome deletion and tumor development. $5X10^6$ of Phoenix-Eco packaging cells were transfected with 15 μg of LacZ/Neo-5'Pur-LoxP-TK or Hyg-3'Pur-LoxP through the calcium phosphate precipitation method. Forty eight hours after the transfection, LacZ/Neo-5'Pur-LoxP-TK viral supernatant was added to $5x10^5$ NIH3T3 or NOG8 cells with 8ug/ml of polybrene. After 5 hours of incubation, the medium with retrovirus was replaced with fresh medium. Hyg-3'Pur-LoxP retrovirus was used for

infection on the second day. At 48 h after retroviral infection, the cells were subjected to G418 and hygromycin selection. A total of 20 plates of NOG8 cells were used.

One week after the selection, each plate was infected with 10^8 adenovirus expressing Cre recombinase (provided by Dr. Graham from NIH). After 12 hours, the cells were washed with PBS three times and fresh medium was added. The following day, 2 μg/ml of puromycin was added to the medium and the cells were selected with puromycin for five days. Eight-week-old BALB/c athymic nude mice were injected subcutaneously with $1X10^6$ cells from each plate. Two months later, the tumor was isolated. Half of the tumor was used for DNA preparation and half of the cells were used to establish the tumor cell line.

Inverse-PCR and sequencing. To determine the retroviral integration site, 5 μg of genomic DNA from the tumor was digested with BamHI plus ApoI for localization of the 3'LTR or SSPI plus Afl III for localization of the 5'LTR. The digested DNA was then blunted with T4 polymerase, circularized by dilution and ligation using T4 DNA ligase in a total volume of 500 μl at 16^oC for 18 hours. Circular DNA was purified and used in the primary PCR reaction using primers derived from the retroviral vectors. 0.1 μl of primary PCR product was used as template for secondary PCR with nested primers. The secondary PCR product was sequenced at the Northwestern University Biotech facility. The primary and nested primers used were as follows:

Localization of the 5'LTR: 5'-CATGGTCAGGTCATGGATGA-3' and 5'-AGGAACAGCGAGACCACGATT-3'; its nested primers: 5'-ACGATGGTGCAGGATATCCT-3' and 5'-GATGCAAACAGCAAGAGGCT-3'.

Localization of the 3'LTR: 5'-CCGCTAAAGCGCATGCTCCA-3' and 5'-TGCAAGAACTCTTCCTCACG-3'; its nested primers: 5'-CTGCCTTGGGAAAAGCGCCT-3' and 5'-CTCGACATCGGCAAGGTGT-3'.

Semi-quantitative RT-PCR. Semi-quantitative RT-PCR was carried out with the SuperScript one-step RT-PCR kit from Invitrogen. One μg of total RNA was reversely transcribed with SuperScript II reverse transcriptase, and then amplified by a PCR consisting of 35 cycles with denaturing at 94 ^oC for 15 sec, annealing at 55 ^oC for 30 sec, and extension at 68 ^oC for 1 min. For each reaction, 10 μCi of $[^{35}S]$-dATP was added and 20 μl of amplified product was taken out at cycles 25 and 30. A 5 μl aliquot of PCR product was resolved on PAGE, which was then exposed to X-ray film. The primers used were as follows:

β-actin: 5'-CCATCTACGAGGGCTATGCT-3' and 5'-GCAAGTTAGGTTTTGTCAAAGA-3'.

Perp: 5'-AACCACATCCAGACATCGTC-3' and 5'-GTTTCCTCCTCAGATCCATC-3'

HGF: 5'-GAACTCTGCAGATGAGTGTG-3' and 5'-GGAATGTCACAGACTTCGTA-3'

MMP2: 5'-GAAGTGACTGGGCATGATCT-3' and 5'-ATGCTGCCTTTAACTGGAGT-3'

mFat4:5'-CCAACGCTCTGGTCACGTAT-3' and 5'-CTCCATTCACACCAGAGTCA-3'

Human and mouse fusion Fat4:5'-ATCGACCACTGAATTGACCA-3' and 5'-CTCCATTCACACCAGAGTCA-3'.

hFat4: 5'-TATCACAAAACGCCCTTGCT-3' and 5'-TGGATTGTCATTGATATCCTG-3'

Methylation assay. Sodium bisulfite DNA treatment was performed as described previously (14). In brief, 1μg of genomic DNA (10 μl) was denatured by adding an equal volume of 0.6 N NaOH for 5 min, followed by the addition of 208 μl of 3.6 M sodium bisulfite and 12 μl of 10 mM hydroxyquinone. This mixture was incubated at 55°C for 16 h to convert cytosine to uracil. Treated genomic DNA was subsequently purified using the Wizard clean up system (Promega), precipitated with ethanol, and resuspended in 100 μl of distilled H_2O.

PCR was performed in a 50-μl reaction using 5 μl of sodium bisulfite-treated DNA. After the PCR, a nested PCR was performed and the final PCR product was sequenced directly. The primers for PCR and corresponding nested PCR were as follows:

Mouse Fat4 promoter: 5'-GGTATGGTGAGGGGAGGGGA-3' and 5'-CTAAATTTCGAAAATCCGAAAAAC-3'; its nested primers: 5'-GGCGTTGAGGAGGAAGGGAAA-3' and 5'-CAAAAAACTTTAAAACTTACCCC-3'.

Human Fat4 promoter: 5'-AATAAATTCTAAAATTTCTAAAAAC-3' and 5'-GTTAGTAGTTTTGTTTGGTGTTA-3'; its nested primers: 5'-ACTTCTCCCAACTCTCATCC-3' and 5'-GATAAAGAGAAGGAAGGGGTG-3'.

Transfection. The BAC clone DNA (RPCI-23 MM BAC 194E5 and RPCI-11 HS BAC 15017 from Invitrogen) were linealized with Not I, mixed and treated with ligase for 2h at 16^{o}C. Tumor cells ($3X10^5$) were seeded on 10cm plates 24 h before transfection. Cells were transfected for 5 h with 15μg of BAC clone DNA and 0.1ug of Plib-Bla which carries the blasticidin resistance gene using Lipofectamine Plus reagent (Invitrogen). Blasticidin (10μg/ml) was added into the culture medium 48h later. The individual clones were picked up 7 days later and expanded. The mRNA expression of human and mouse fusion Fat4 in these clones was examined by RT-PCR.

Northern blot. Total RNA (30 μg) isolated from NOG8 or tumor cells with TRIzol reagent (Invitrogen) was separated by electrophoresis on a 1% agarose/formaldehyde gel and transferred to a Nytran membrane. [α-^{32}P]dCTP-labeled mFat4 cDNA probe was prepared with random prime labeling kit (Invitrogen). Standard pre-hybridization, hybridization, and washing procedures were performed, and the membrane was exposed to X-ray film for 48h.

In vivo tumorigenicity assay. Two clones expressing Fat4 and two clones not expressing Fat4 were used for the tumorigenicity assays in vivo. The selected clones, $1X10^6$ cells from each, were injected subcutaneously into 8-week-old BALB/c athymic nude mice. The maximal tumor diameter was determined by caliber measurements once a week up to sixth week.

Collagen gel culture. Rat tail collagen gel solution in glacial acetic acid was neutralized with solution containing sodium bicarbonate on ice. NOG8 or tumor cells ($1X10^5$) were then mixed with collagen gel and plated in the 24-well plates which had been covered with a “bottom” layer of collagen gel. After gelation was complete, the gel was overlayed with culture medium. The formation of duct-like structure was examined 3 days later.

Microarray assay. Total RNA was isolated from NOG8 cells, tumor cells, tumor cells expressing Fat4 using TRIzol (Invitrogen) and then cleaned with an SV total RNA isolation kit (Promega) in accordance with the manufacturer's instructions. The integrity of RNA was confirmed by the presence of sharp 28S and 18S bands on a denaturing agarose gel. RNA was submitted to the Northwestern University Microarray Core Facility for cDNA and cRNA synthesis, labeling and hybridization with Affymetrix mouse genome 430 2.0 arrays according to the manufacturer's instructions. Microarray data analysis was performed with Affymetrix microarray software. For each gene, an indicator of its expression level was given as present, absent, or marginal call. Transcripts were defined as up-regulated or down-regulated only when identified as "present". The -fold change between NOG8 and tumor cells had to be at least 3-fold to designate a transcript as being differentially expressed.

RESULTS

Creation of random chromosome deletion. In an attempt to make random chromosome deletion, we have designed two retroviral vectors (Fig. 1). To generate the vectors, an artificial intron with LoxP sequence was introduced into the puromycin resistance gene and tested in mammalian cells to make sure that the puromycin resistance gene with the intron was still functioned. The puromycin resistance gene was then divided into two parts: 5’part with LoxP and 3’part with LoxP, which were cloned into vector LacZ/Neo-5’Pur-LoxP-TK and Hyg-3’Pur-loxP respectively. With the intron, the recombinant between these two vectors can generate an intact puromycin resistance gene with the correct reading frame. In addition, vector LacZ/Neo-5’Pur-LoxP-TK contained the LacZ/Neo gene conferring resistance to G418, and the TK gene which provided a negative selection with gancyclovir, while vector Hyg-3’Pur-LoxP possessed hygromycin resistance gene. After the integration of the two retroviruses, the cells were infected with adenoviruses expressing Cre recombinase, which mediates the recombination between two LoxP sites. Cells with two retroviruses integrated on the same chromosome and in the same orientation would generate a deletion due to the recombination. Translocation can occur when two retroviruses are

integrated on two different chromosomes. Cells with the recombination carried an intact puromycin resistant gene which allowed for the selection by puromycin.

We tested the system with NIH3T3 cells. Six clones were isolated and their retroviral integration sites were determined through Inverse-PCR and sequencing. Three of the six clones contained deletions of: 1.2Mbp on chromosome 4 (from 102.6Mbp to 103.8Mbp), 7.5Mbp on chromosome 5 (from 134.6Mbp to 142.1Mbp), and 19.5Mbp on chromosome 10 (from 24.6Mbp to 44.1Mbp), demonstrating that large chromosome deletions could be effectively generated with our system. The other three clones contained translocations which were translocations between chromosome 1 and 19, chromosome 3 and 6, and chromosome 2 and 3. Due to the lower efficiency of recombination between LoxP sites on different chromosomes than those on the same chromosome, we found that equal number of deletions and translocations were obtained although the chance of integration on different chromosomes is far more than that on the same chromosome.

Identification of Fat4 as a candidate tumor suppressor gene. NOG8 cells are diploid, immortal mouse mammary epithelial cells isolated from mammary epithelial cell line NMuMG (37). When injected subcutaneously into nude mice, we found that NOG8 cells failed to produce tumors in three months (data not shown). A total of 20 plates of NOG8 cells were sequentially infected with the LacZ/Neo-5'Pur-LoxP-TK virus, Hyg-3'Pur-loxP virus and Cre adenovirus. Cells from each plate (1 X 10^6) after the selection with puromycin were injected subcutaneously into one site of the nude mice. Two months later, 9 tumors developed and were isolated from the mice.

For one tumor, Southern blot revealed that only a single retroviral vector presented in the tumor (data not shown). Inverse –PCR and sequencing revealed that the 5'LTR of the retrovirus was located at 37,747kb on chromosome 3 while the 3'LTR was found to be at 40,623 Kb on the same chromosome. Therefore, a ~3Mbp fragment of chromosome 3 from 37,747 kb to 40,623 kb was deleted in this tumor (Fig. 2a). Histology revealed that the tumor was a poorly differentiated carcinoma with occasional gland differentiation (Fig. 2b).

By looking into the mouse genome map, we identified 14 genes within the ~3M bp deleted region. As the first step to linking any of the genes to the tumorigenesis, we examined the expression of these 14 genes in the tumor and wild type NOG8 cells by semi-quantitative RT-PCR. We found that Fat4 mRNA level was substantially decreased in the tumor (Fig. 2c) while the mRNA of the other 13 genes remained unchanged (data not shown). As only partial Fat4 cDNA sequence and hypothetical Fat4 coding sequence derived from the mouse genomic sequence using gene prediction method were available in Genbank, we determined the exact mouse Fat4 cDNA through RT-PCR and sequencing (Genbank No: DQ286572). The Fat4 gene turned out to encode a protein with 4981 amino acids, which contains 5 epidermal growth

factor (EGF) domains and 2 laminin-A-G domain repeats in addition to the 34 cadherin repeats in the extracellular domain.

Mutations in the mRNA lead to instability, resulting in decreased mRNA levels, thus, we searched for any mutations in the Fat4 gene of the tumor by sequencing. We did not detect any nonsense mutations in the Fat4 exons or any mutations which could disrupt the mRNA splicing (data not shown). A CpG island encompassing about 2.5 kb was found in the mouse Fat4 gene promoter region. We then examined the Fat4 promoter to see if it was methylated. We amplified and sequenced the promoter region of Fat4 using sodium bisulfite-treated genomic DNA prepared from tumor cells. Indeed, the vast majority of CpG sites (20/22) were methylated in the tumor cells while the CpG sites in the wild type NOG8 cells were not methylated (Fig. 3A). When tumor cells were treated with the "demethylating" agent 5-aza-2'-deoxycytidine for 48 h, the expression of Fat4 mRNA was dramatically increased (Fig. 3B), indicating that methylation of the Fat4 promoter silenced the expression of Fat4 from the second allele following the deletion of the first Fat4 allele.

To confirm that the loss of expression of Fat4 is indeed involved in the tumorigenesis of NOG8 cells, we expressed the Fat4 gene in the tumor cells to see if Fat4 could suppress the tumor formation in nude mice. A BAC clone containing the promoter region, first exon, and part of the first intron of the human Fat4 gene, and another BAC clone containing part of the first intron and all exons except exon 1 of the mouse Fat4 gene were then cotransfected along with a blasticidin resistance gene into cells derived from the tumor. The expression of the Fat4 gene in individual cell clones was examined with RT-PCR using a primer specific for the human Fat4 in the first exon and a second primer specific for mouse Fat4 in the second exon (Fig. 4A). The RT-PCR product was sequenced to ensure that the reading frame was not altered due to inappropriate splicing of the first intron (data not shown). The Fat4 expression in these clones were further confirmed by Northern Blot (Fig. 4B). Two clones expressing the Fat4 gene, and two clones not expressing the Fat4 gene were selected for further studies. Cells from each of the selected clones ($1X10^6$) were injected subcutaneously into nude mice. As shown in Fig. 4C, tumor cells expressing Fat4 grew much slower than those not expressing Fat4, confirming that Fat4 expression inhibited the growth of tumor cells in nude mice (Fig. 4C).

Substantially decreased or no expression of Fat4 mRNA in about 60 % of breast cancer cell lines and primary breast tumors. We examined Fat4 mRNA in 23 primary breast cancer samples and 10 established breast cancer cell lines. A substantial decrease or absence of Fat4 mRNA was observed in 14 of 23 primary breast cancers. Representative data from this analysis are shown in Fig. 5A. Of the 10 breast cancer cell lines examined (BT474, T47D, BT20, ZR-75-1, MDA-MB-134-V1, MDA-MB-157, MDA-MB-231, MDA-MB-453, MCF-7, and MDA-MB-468), 6 cell lines, namely BT474, T47D, BT20,

ZR-75-1, MDA-MB-134-V1, and MDA-MB-468, revealed markedly decreased or no expression of Fat4 mRNA (Fig., 5A for representative data).

Like the mouse Fat4 promoter region, the human Fat4 promoter region also contains a CpG island spanning about 2.5 kb. To understand the mechanism underlying the down-regulation of Fat4 mRNA, we examined the methylated status of CpG sites in the Fat4 promoter from four cell lines. BT20 showed complete methylation in the ten CpG sites examined, and BT474 had partial methylation (4/10), whereas T47D, just like MCF7 which expresses Fat4, had no methylation at all (0/10) (Fig.5B). Therefore, methylation of the Fat4 gene promoter is associated with the silenced expression of Fat4 in some of the breast cancer cell lines, and other mechanisms such as gene regulation could play a role in other cell lines.

Fat4 deficient tumor cells could not form a duct-like structure in collagen gel. When suspended in collagen gels, NOG8 cells, just like primary mouse epithelial cells (46), developed an extensive ductlike structure (Fig.6), which is reminiscent of the branching morphogenesis of epithelial ducts in mammary gland. In contrast, the tumor cells proliferated as individual cells without branching (Fig.6), indicating that Fat4 could be involved in the duct formation, similar to the Fat-like gene in drosophila (6).

Identification of the genes whose expression were down or up-regulated with Fat4 deficiency. To understand the mechanism by which Fat4 deficiency led to the tumorigenesis of NOG8 cells, a microarray analysis was performed to identify the genes, expression of which are affected by Fat4 expression. Given that tumor cells might acquire additional genetic or epigenetic changes following Fat4 downregulation during tumor expansion, gene profiling was also done with tumor-derived cells expressing the Fat4 gene. We expected that genes regulated by Fat4 should be those expression of which was down or up-regulated in tumor cells but unchanged in tumor derived cells expressing Fat4. We identified a total of 36 genes which were down-regulated in tumor cells compared with NOG8 cells and tumor cells expressing Fat4, including TP53 apoptosis effector (Perp) (Table1). As expected, Fat4 was dramatically down-regulated in tumor cells. We also identified a total of 22 genes which were upregulated in tumor cells, including metalloproteinase 2 (MMP-2) and Hepatocyte growth factor (HGF) (Table 2). The altered expression of Perp, MMP-2, and HGF were further confirmed with semi-quantitative RT-PCR (Fig.7).

DISCUSSION

In an effort to isolate novel tumor suppressor genes from breast cancers, we developed a random chromosome deletion method based on the Cre-LoxP system. The creation of large chromosome deletions with this method was successfully tested in NIH3T3 cells. The mouse genome has 2.5 $X10^9$ bp DNA which is distributed on 20 chromosomes. If the deletion is 2000 kb and any region in the mouse genome has a 95% probability of deletion, the required number of the deletions according to the statistics based on

the Poisson distribution are: $LN(1-0.95)/LN[1-2x10^6/2.5x10^9)]= 3.74 \ x10^3$. Therefore, only a pool of several thousand mouse cells with chromosome deletions need to be generated to have deletions covering the whole genome. With the first allele of the candidate tumor suppressor gene deleted, inactivation of the second allele can be achieved by treating the cells with a mutagen, natural occurring point mutation, or an epigenetic modification, resulting in tumorigenesis. Following selection for tumor cells, the tumor suppressor gene can be identified through determining the chromosomal deletion region in the cells.

We applied this method to an immortalized mammary epithelial cell line NOG8 cells. In addition to the ~3Mbp deletion described above, two distinct deletions were identified from the 9 tumors. One deletion containing the tumor suppressor gene BRCA1 spanned ~20Mbp on mouse chromosome 11 (from 83Mbp to 103Mbp). We are currently investigating to see if that mutation of BRCA1 is responsible for the tumorigenesis of NOG8 cells. The third deletion spanned 12.6Mbp on chromosome 14 (from 49Mbp to 61.6 Mbp). The remaining 6 tumors had chromosome translocations instead of deletion (data not shown). For those cells which did not develop tumors, we are trying to induce the tumor development by treating the cells with a mutagen in hopes that the mutagen will inactivate the other copy of a potential tumor suppressor gene. One major challenge using this method is to locate the tumor suppressor gene from a 10 to 20Mbp of chromosome fragment, although it can be facilitated by the identification of recurrent and overlapping deletions. Nevertheless, we demonstrated that the random chromosome deletion method is a powerful method to identifying tumor suppressor genes, which can be applied to other type of tumors.

We identified Fat4 in the deleted ~3M bp region as a strong candidate for a breast tumor suppressor gene. There are four members of Fat family in mice and humans, which share high homology to the *Drosophila* Fat gene. Just as other membrane proteins, the cytoplasmic regions of Fats are expected to play an important role in the signaling transduction. However, we found that the cytoplasmic regions from Fat1, Fat2 and Fat3 exhibited no homology to that of the *Drosophila* Fat protein. This may explain the observation that unlike the *Drosophila* Fat, the null mutation of Fat1 in mice did not show overgrowth of any organs. Instead, several small regions from the cytoplasmic region of Fat4 are highly conserved in the *Drosophila* Fat gene (Fig. 8), suggesting that only Fat4 is the homologue of *Drosophila* Fat gene.

In addition to point mutations, epigenetic modifications such as gene methylation have been found to be a cause of inactivation of tumor suppressor genes in tumors (3, 13, 18). The mouse Fat4 gene promoter region contains a CpG island which was methylated in the tumor cells and Fat4 could re-express with the treatment of a demethylating agent. Therefore, the second allele of Fat4 in the NOG8 cells was inactivated through promoter methylation following the deletion of the first Fat4 allele. While how the gene methylation is regulated and how loss of an allele triggered the methylation of the other allele

remains unknown, many other tumor suppressor genes could be inactivated in this way as loss of heterozygosity is very common in almost all types of tumors.

We found that loss of Fat4 expression occurred to a large fraction of both primary breast tumors and breast cell lines, implicating its important role in breast tumorigenesis. Methylation of Fat4 gene promoter was found to be associated with the silenced expression of Fat4 in some of the breast cancer cell lines. As loss of Fat4 expression in breast carcinoma also occurred in the absence of promoter methylation, it is important to characterize the mechanism underlying the transcriptional regulation of the human Fat4 gene, which could be involved in the decreased expression of Fat4 in breast tumors. In addition to promoter methylation and transcriptional regulation, a mutation could potentially inactivate the Fat4 gene. We are currently examining a large number of specimens to identify the potential mutation. As the loss of heterozygosity in the chromosome 4q28, where the human Fat4 gene is located, was observed in several cancers including colon, prostate lung, and liver (7, 17, 22, 23, 28), Fat4 could be involved in other types of tumors.

Our microarray assay revealed that TP53 apoptosis effector (Perp) was down-regulated, and matrix metalloproteinase 2 (MMP-2) and Hepatocyte growth factor (HGF) were upregulated with the Fat4 deficiency, which was confirmed by semi-quantitative RT-PCR. Perp is a p63 regulated gene essential for epithelial integrity through promoting the stable assembly of desmosomal adhesive complexes (1, 20, 21,). The down-regulation of Perp might contribute to the failed formation of a duct-like structure of Fat4 deficient tumor cells in collagen gel. MMP-2 is a member of the matrix metalloproteinases (MMPs) family which is structurally related to zinc-dependent endopeptidases and can degrade extracellular matrix components (27, 38). MMP-2 degrades type IV collagen, which is a major component of base membranes (47). MMP-2 expression has been correlated with tumor invasion and metastasis in various cancers including breast cancers (2, 9, 35). HGF is a multi-functional cytokine which stimulates morphogenesis, cell survival, mitogenesis, motility, invasion, and metastasis (5, 16). Over-expression of HGF is found in many types of invasive cancers including breast cancers (34, 41, 43). The altered expression of these genes due to Fat4 deficiency suggest that the loss of Fat4 expression released the cells from neighbor cells, induced the expression of potent cytokines, leading to the tumorigenesis.

The evidence supporting Fat4 as a tumor suppressor gene is the loss of Fat4 in nontumorigenic mammary epithelial cells transformed the cells into tumorigenic cells and restoring Fat4 in these tumor cells markedly inhibited the tumorigenecity. These studies were done with a mammary epithelial cell line which had been immortalized. It is important to understand the role of Fat4 in mammary epithelial tumorigenesis under physiological conditions. As a tumor suppressor gene, Fat4 could be an initiating factor during mammary tumorigenesis or a factor involved in tumor progression. A definitive conclusion can be reached through the establishment of an animal model with Fat4 deficiency in mammary glands.

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FIGURE LEGENDS

Fig. 1. The chromosome deletion by the LoxP-Cre system. The cells are infected with two retroviruses containing LoxP sites. If the two retroviruses are integrated on the same chromosome and the two LoxP sites are in the same direction, the region between the two LoxP sites is deleted by expression of Cre recombinase and an intact puromycin resistance gene is created. The cells with successful recombination are selected by treatment of puromycin.

Fig. 2. (A). The deleted region was identified to be ~3Mbp on mouse chromosome 3. (B). The histology of the tumor with deletion on chromosome 3. (C). Loss of Fat4 expression in the tumor. Semi-quantitative RT-PCR was performed with addition of ^{35}S-dATP. For each PCR reaction, aliquots of 5 μl of PCR product at 25 cycles, 30 cycles, and 35 cycles were resolved on PAGE. β-actin served as the control.

Fig. 3. (A). The methylation state of the CpG island in NOG8 cell and tumor cells. The CpG map of the Fat4 promoter region is shown with the analyzed region of the CpG island (370bp) indicated. The indicated promoter region containing 22 CpG sites was analyzed by direct sequencing after amplification of sodium bisulfite-treated genomic DNA. A closed circle indicates a methylated CpG and an open circle indicates an unmethylated CpG. (B). Increased expression of Fat4 gene in tumor cells with the treatment of 5-aza-2’-deoxycytidine(5-Aza-dC). Tumor cells were treated with 5 mM 5-aza-2’-deoxycytidine(5-Aza-dC) for three days. The expression of Fat4 was examined by semi-quantitative RT-PCR.

Fig. 4. Expression of the Fat4 gene inhibited the growth of a tumor in nude mice. (A). The mRNA expression of human and mouse fusion Fat4 gene as revealed by RT-PCR, which was performed with one primer specific for human Fat4 in the first exon and a second primer specific for mouse Fat4 in the second exon. The RT-PCR product was sequenced to ensure that the reading frame was not altered due to inappropriate splicing of the first intron (data not shown). Tumor Clone 3 and 4 expressed Fat4 while Clone 1 and 2 did not express Fat4. (B). The mRNA expression of the Fat4 gene examined by Northern blot. Fat4 mRNA was detected in tumor Clone 3 and 4 but not in Clone 1 and 2, consistent with the result from the RT-PCR. (C). The curve of tumor growth in nude mice. Two clones of tumor cells not expressing Fat4 or two clones of tumor cells expressing Fat4 ($1X10^6$ cells from each clone) were injected into nude mice, respectively. The experiment was performed in triplicate and the average tumor sizes

were calculated. The tumors from clones expressing Fat4 were much smaller than that from tumor cell line not expressing Fat4.

Fig. 5. (A). Decreased expression of Fat4 gene in some of the breast cancers. Total RNA was made from breast tumor cell lines or primary tumors. Semi-quantitative RT-PCR was performed with the addition of S^{35}-dATP. Tumor 1 and cell line BT20 did not express Fat4 while Tumor 2 and cell line MDA-MB-231 expressed Fat4. (B). Methylation of the CpG island in the Fat4 promoter region in breast cancers. A CpG island spanning ~2.5 kb was located in the promoter region and first exon of the human Fat4 gene. The fragment containing 10 CpG sites was indicated with arrows and examined in breast cancers cell lines. The methylated state for four tumor cell lines was shown, with an open circle representing an unmethylated CpG site and a close circle representing a methylated CpG site.

Fig. 6. NOG8 cells but not tumor cells formed duct-like structure in collagen gel. NOG8 cells or tumor cells ($1x10^5$) mixed with the collagen gel were inoculated in a six-well plate. The images were taken three days later.

Fig 7. Decreased Perp expression, and increased HGF and MMP-2 expression in Fat4 deficient tumor cells compared with NOG8 cells and tumor cells expressing Fat4. Semi-quantitative RT-PCR was performed with addition of S^{35}-dATP.

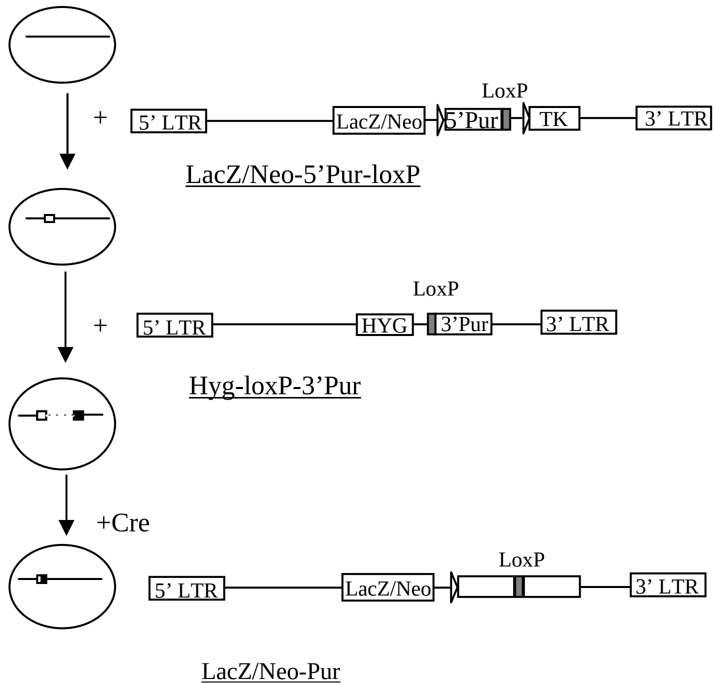
Fig 8. Homology of the cytoplasmic region at the C-terminus between the mouse Fat4 (mFat4) and the *D. melanogaster* Fat (dFat). Pluses represent similar amino acids. Minuses represent space inserted for optimum alignment.

Table 1. Genes with decreased expression in tumor cells

GeneBank No.	Gene Name	Fold(Tumor/NOG8)
AV026492	Thrombospondin 1	0.0080
BB536078	Fat4	0.018
NM_007987	Fas antigen	0.022
NM_022032	Perp	0.038
BC010816	Lims2	0.039
BB812574	G protein-coupled receptor 126	0.046
AW125230	Enpp2	0.046
BB820441	Nfkbie	0.051
D17546	Collagen	0.055
AK005150	SSDP	0.059
NM_013738	Pleckstrin 2	0.062
NM_017465	Sult2b1	0.066
NM_009929	Col18a1	0.070
NM_134159	Interleukin 17 receptor-like	0.082
AK007630	Cyclin-dependent kinase inhibitor 1A	0.089
NM_011125	Phospholipid transfer protein	0.10
BB711990	Transmembrane and coiled coil domains 3	0.10
NM_008471	Keratin complex 1, acidic, gene 19	0.11
AF128193	Small inducible cytokine A7	0.12
AV298805	Fhod1	0.13
BB528350	Syndecan 3	0.13
NM_009141	Scyb5	0.13
NM_013473	Annexin A8	0.15
NM_010145	Epoxide hydrolase 1, microsomal	0.16
AF189769	Beta adducing	0.16
NM_021515	Adenylate kinase 1	0.17
BE652876	Fatty acid desaturase 3	0.22
BB530223	Putative phosphatase	0.24
NM_009291	Stimulated by retinoic acid gene 6	0.24
BM233698	Colony stimulating factor 1	0.24
NM_019645	Plakophilin 1	0.25
BC006713	Diacylglycerol kinase, alpha	0.27
NM_008176	GRO1	0.27
NM_010358	Glutathione-S-transferase, mu 1	0.28
AK018622	Ralgps2	0.31
U03561	HSP27	0.32

Table 2. Genes with increased expression in tumor cells

GeneBank No.	Gene Name	Fold(Tumor/NOG8)
NM_007423	Alpha fetoprotein	100.8
BC007144	Pscdbp	88.0
NM_010743	Interleukin 1 receptor-like 1	79.6
D13695	ST2L protein	78.1
BB482899	Basonuclin	59.4
BF147716	Matrix metalloproteinase 2	48.9
BB782729	SLIT and NTRK-like family, member 5	31.4
NM_011157	Proteoglycan, secretory granule	17.2
AA038464	Amyotrophic lateral sclerosis 2	9.9
AF071068	Aromatic-L-amino-acid decarboxylase	6.2
BB667762	Butyrylcholinesterase	5.9
AF042856	Hepatocyte growth factor	5.6
BB817939	Transient receptor protein 1	5.6
NM_027884	Tensin	4.2
U95030	CD166	4.1
NM_053128	Protocadherin beta 3	3.9
BB503614	Cytohesin binding protein	3.5
BC017678	Rgl1	3.3
AY075132	Helicard	3.3
AI481797	Interferon activated gene 205	3.3
BF451748	Telokin	3.2
BF457392	Phosphatidic acid phosphatase type 2c	3.1



LoxP
+
5' LTR
LacZ/Neo
5'Pur
TK
3' LTR
LacZ/Neo-5'Pur-loxP
LoxP
+
5' LTR
HYG
3'Pur
3' LTR
Hyg-loxP-3'Pur
+Cre
LoxP
5' LTR
LacZ/Neo
3' LTR
LacZ/Neo-Pur

Fig. 1

A.

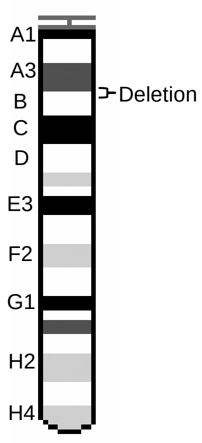


Fig. 2A

B.

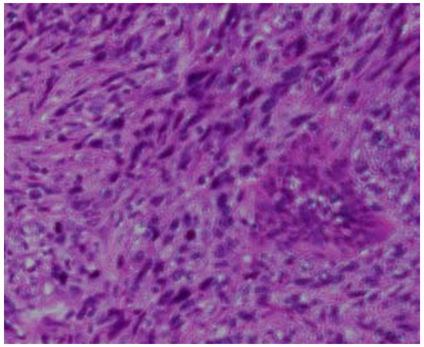


Fig. 2B

C.

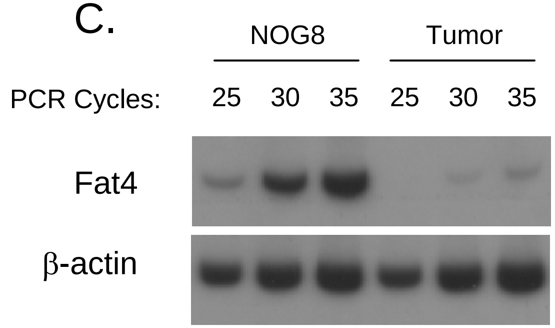


Fig. 2C

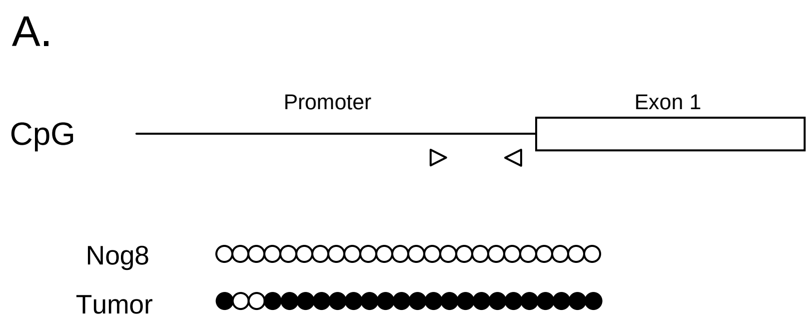


Fig. 3A

B.

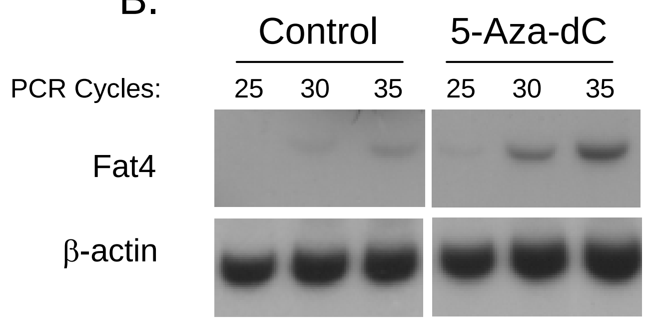


Fig. 3B

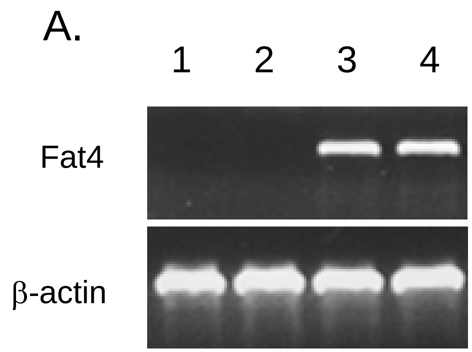


Fig. 4A

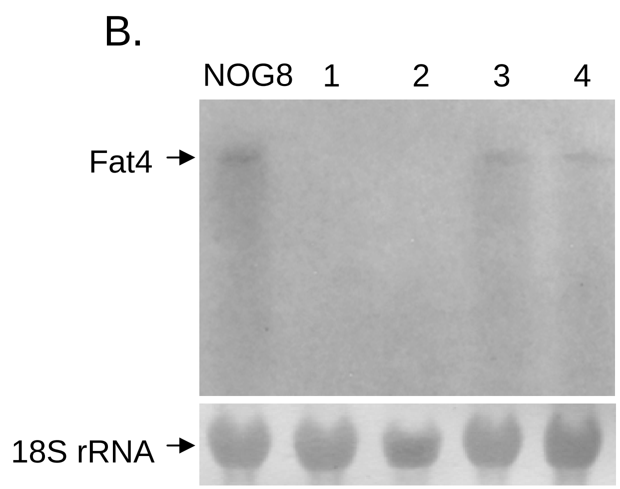


Fig. 4B

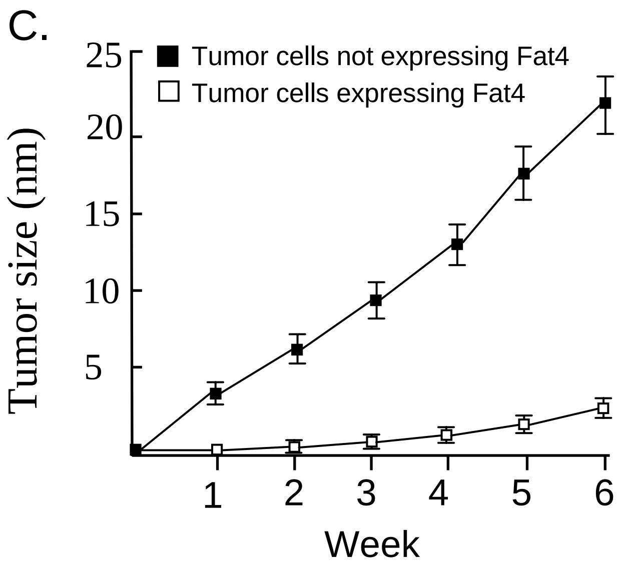

C.
25
20
15
10
5
Tumor size (nm)
Tumor cells not expressing Fat4
Tumor cells expressing Fat4
1
2
3
4
5
6
Week

Fig. 4C

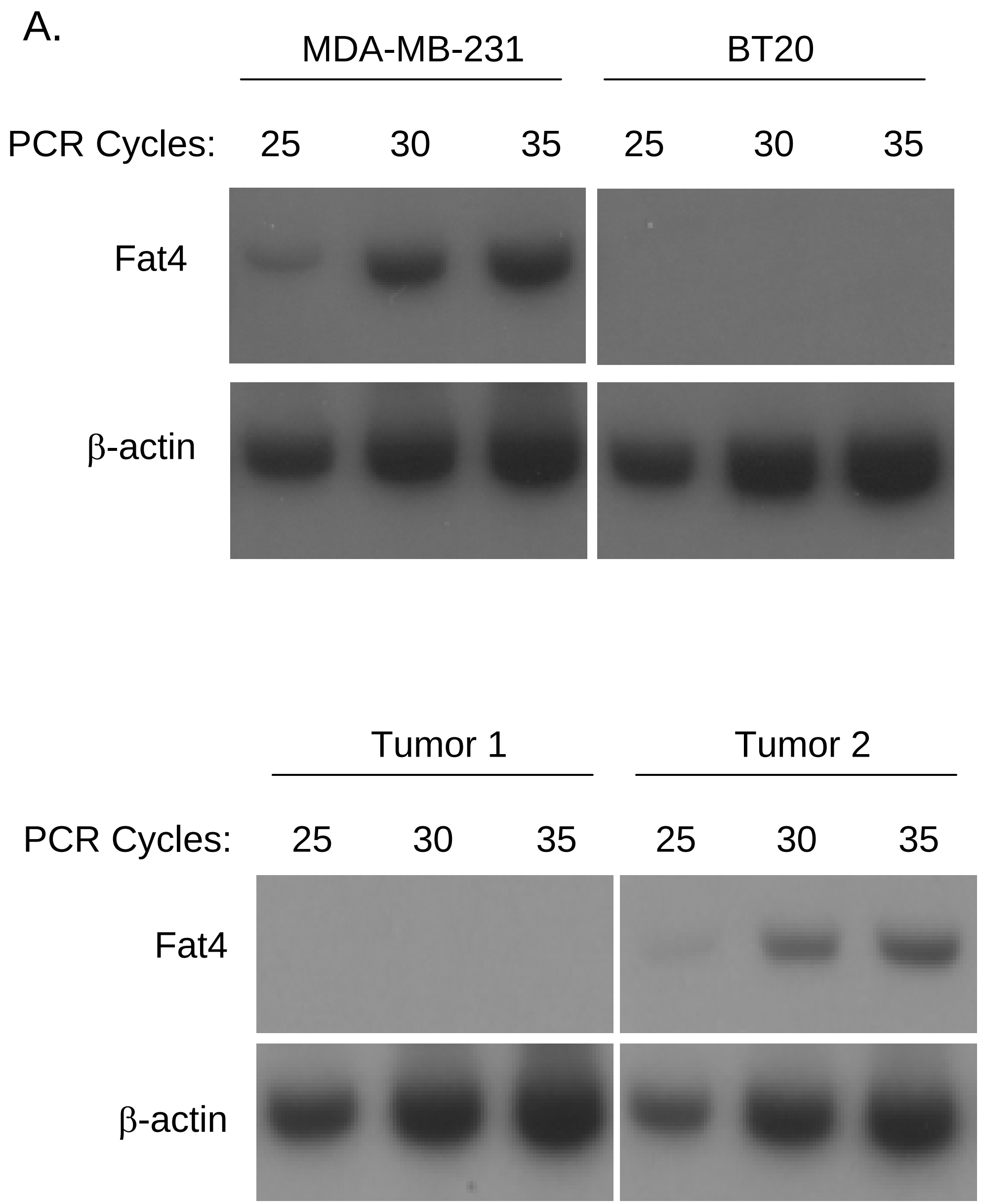

A.
MDA-MB-231
BT20
PCR Cycles: 25 30 35 25 30 35
Fat4
β-actin
Tumor 1
Tumor 2
PCR Cycles: 25 30 35 25 30 35
Fat4
β-actin

Fig. 5A

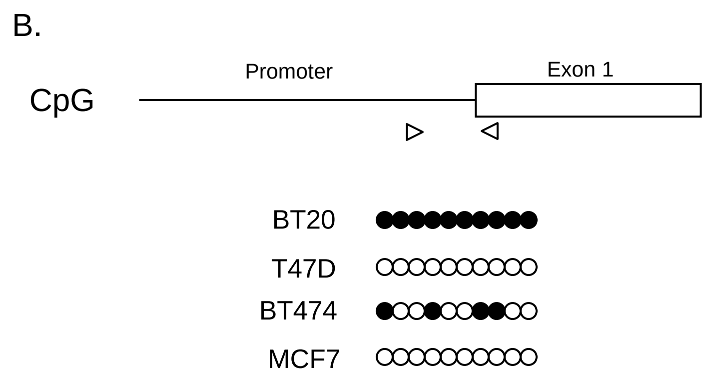


Fig. 5B

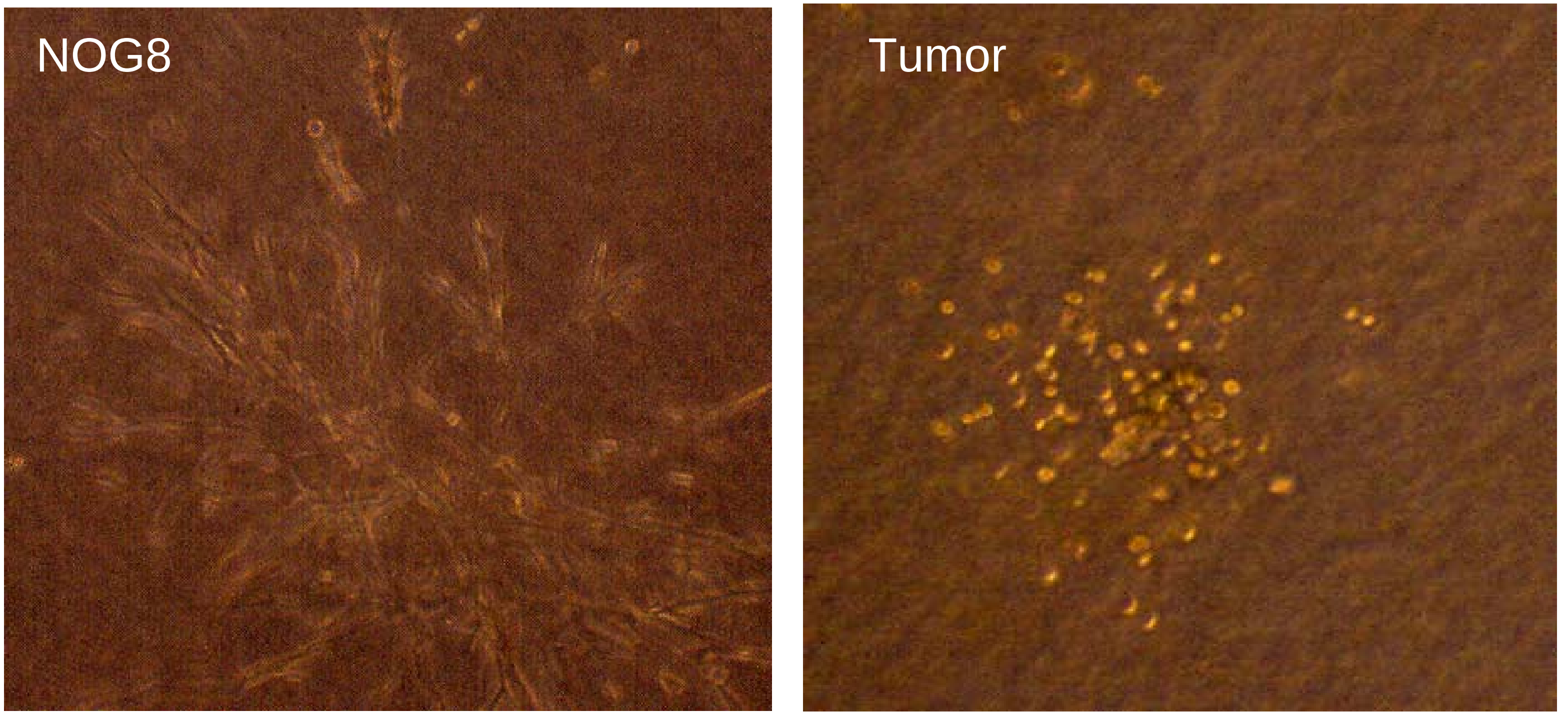


Fig. 6

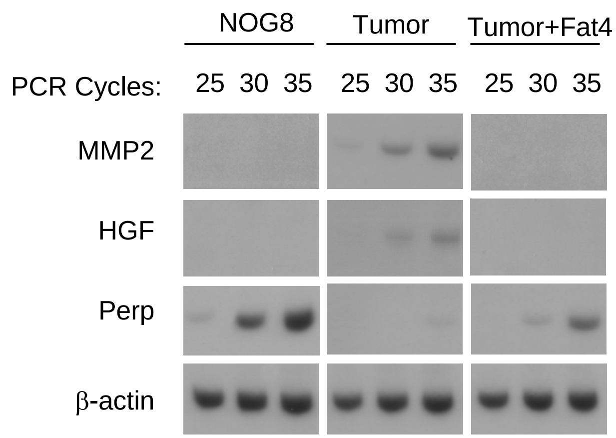

NOG8
Tumor
Tumor+Fat4
PCR Cycles:
25 30 35 25 30 35 25 30 35
MMP2
HGF
Perp
β-actin

Fig. 7

```
mFat4  4573  RKQPEGNPKPDIIERENPYLIFDETDIP----HNSETIPSAPLASPEQEIEHYDIDNASS  4628
             ++Q +   +PDIIERE+P LI ++  +P    H      ++ +    +  EHYD++NASS
dFat   4696  QQQQQRPQRPDIIERESP-LIREDHHLPIPPLHPLPLEHASSVDMGSEYPEHYDLENASS  4754

mFat4  4629  IAPSDADIIQHYKQFRS----HTPKFSIQRHSPLGFARQSPMPLGASSLTYQPSSYGQGL  4684
             IAPSD DI+ HYK +R        K S+    P+            S    Q   +
dFat   4755  IAPSDIDIVYHYKGYREAAGLRKYKASV---PPVSAYTHHKHQNSGSQQQQQQHRHTAPF  4811

mFta4  4685  RTSSLSHSACPTPNPLSR-HSPAPFSKPSAFYRNSPARELHLPLRDGGTLEMHGDPCQPG  4743
              T +      P P   SR H   P ++ S     S  +   L L D     + G P Q
dFat   4812  VTRNQGGQPPPPPTSASRTHQSTPLARLSPSSELSSQQPRILTLHD-----ISGKPLQSA  4866

mFat4  4744  MFNYATRLG-----------RRSKSP-QAMASHGSRPGSRLKQPIAQIPLESSPPVGLSI  4791
             +    +  G           R   SP  +  S  S   SR K  + Q   + +  +GL+
dFat   4867  LLATTSSSGGVGKDVHSNSERSLNSPVMSQLSGQSSSASRQKPGVPQQQAQQT-SMGLTA  4925

mFat4  4792  EEVERLNTPRPRNPSICSADHGRSSSEEDCRRPLSRTRNPADG-IPAPES-SSDSDSHDS  4849
             EE+ERLN  RPR  S+ S     SSS E  R   S       G + A  S S+D   +DS
dFat   4926  EEIERLN-GRPRTCSLISTLDAVSSSSEAPRVSSSALHMSLGGDVDAHSSTSTDESGNDS  4984

mFat4  4850  FTCSEMEYDRE------KPVVYTSRMPKLSQVNESDADDEDNYG--------ARLKPRRY  4895
             FTCSE+EYD        K     S +   S V+ + +  E +            + P  Y
dFat   4985  FTCSEIEYDNNSLSGDGKYSTSKSLLDGRSPVSRALSGGETSRNPPTTVVKTPPIPPHAY  5044

mFat4  4896  HGRRAE-GGPVGTPAAASGAADSTLK---------LGQQAGNFNWDNLLNWGPGFGHYVD  4945
              G  +   G + T  A+     + L              A    W+ LLNWGP + + +
dFat   5045  DGFESSFRGSLSTLVASDDDIANHLSGIYRKANGAASPSATTLGWEYLLNWGPSYENLMG  5104

mFat4  4946  VFKDLASLPE  4955
             VFKD+A LP+
dFat   5105  VFKDIAELPD  5114
```

Fig. 8